Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10847–10851, November 1992
Biochemistry

Nonreplicating vaccinia vector efficiently expresses recombinant genes

(poxvirus/expression vector/attenuation/host restriction)

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Contributed by Bernard Moss, August 20, 1992

ABSTRACT **Modified vaccinia Ankara (MVA), a highly attenuated vaccinia virus strain that has been safety tested in humans, was evaluated for use as an expression vector. MVA has multiple genomic deletions and is severely host cell restricted: it grows well in avian cells but is unable to multiply in human and most other mammalian cells tested. Nevertheless, we found that replication of viral DNA appeared normal and that both early and late viral proteins were synthesized in human cells. Proteolytic processing of viral structural proteins was inhibited, however, and only immature virus particles were detected by electron microscopy. We constructed an insertion plasmid with the *Escherichia coli lacZ* gene under the control of the vaccinia virus late promoter P11, flanked by sequences of MVA DNA, to allow homologous recombination at the site of a naturally occurring 3500-base-pair deletion within the MVA genome. MVA recombinants were isolated and propagated in permissive avian cells and shown to express the enzyme β-galactosidase upon infection of nonpermissive human cells. The amount of enzyme made was similar to that produced by a recombinant of vaccinia virus strain Western Reserve, which also had the *lacZ* gene under control of the P11 promoter, but multiplied to high titers. Since recombinant gene expression is unimpaired in nonpermissive human cells, MVA may serve as a highly efficient and exceptionally safe vector.**

The eradication of smallpox was achieved through immunization with live vaccinia virus (1). Presently, vaccinia virus is used extensively as a gene expression vector and is under evaluation as a recombinant vaccine (2). Because vaccinia virus is infectious for humans, its use in the laboratory has been affected by safety concerns and regulations (3, 4). For general vector applications, health risks would be lessened by the adoption of a highly attenuated vaccinia virus strain. Several such strains were developed for use as safer smallpox vaccines (1). We chose to examine the potential of the modified vaccinia Ankara (MVA) strain as an expression vector because of its extreme attenuation. MVA was derived from vaccinia virus strain Ankara, referred to here as the wild-type virus (WT), by over 570 serial passages in chicken embryo fibroblast cells (CEF) (5). The resulting MVA strain lost the capacity to productively infect mammalian cells and suffered six major deletions of DNA totaling 31,000 base pairs (bp), including at least two host-range genes (refs. 6 and 7; G.S., unpublished data). When tested in a variety of animal species, MVA was proven to be avirulent even in immunosuppressed animals. Most importantly, there is clinical experience using MVA for primary vaccination of over 120,000 humans against smallpox. During extensive field studies, including high risk patients, no side effects were associated with the use of the MVA vaccine (5, 8, 9). However, since MVA cannot replicate in human and most other mammalian cells, high expression of recombinant genes seemed unlikely in view of reports that other host-range vaccinia virus mutants are inhibited early in infection (10, 11).

Contrary to expectations, we found that the expression of late, as well as early, viral genes was unimpaired in human cells despite the inability of MVA to produce infectious progeny. Moreover, recombinant viruses were able to synthesize high levels of a foreign protein in human cells, demonstrating the potential of MVA to serve as an exceptionally safe and highly efficient expression vector.

MATERIALS AND METHODS

Cells and Viruses. The WT Ankara and MVA strains were kindly provided by A. Mayr (Veterinary Faculty, University of Munich). The viruses were routinely propagated and titered in CEF grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Human HeLa and 293 cells were also grown in MEM supplemented with 10% FCS. Virus multiplication was monitored by infecting cell monolayers with 0.05 plaque-forming unit (pfu) per cell. After virus adsorption for 45 min at 37°C, the inoculum was removed; the cell monolayer was washed once with MEM and incubated with fresh medium (MEM containing 2% FCS) at 37°C in a 5% CO_2 atmosphere. At 0, 24, and 48 h postinfection, virus was harvested by freeze-thawing and brief sonication of the infected cells. The resulting lysate was titrated on CEF.

Analysis of Viral DNA. Cytoplasmic DNA from infected 293 cells was transferred to a Hybond N+ membrane (Amersham) with a dot blot apparatus and hybridized to a ^{32}P-labeled MVA DNA probe. Radioactivity was quantitated with a Betascope 603 blot analyzer (Betagen, Waltham, MA). The same DNA preparation from infected 293 cells was also digested with *Bst*EII and separated by electrophoresis on a 1% agarose gel. The DNA was transferred by Southern blot to a Hybond N+ membrane (Amersham) and hybridized with a ^{32}P-labeled probe of the terminal segment of the vaccinia virus genome (12).

Analysis of [^{35}S]Methionine-Labeled Polypeptides. Cell monolayers grown in 12-well plates were infected with virus at a multiplicity of 15 pfu per cell. After adsorption of virus for 45 min at 4°C, MEM supplemented with 2% FCS was added, and the cell cultures were incubated at 37°C in a 5% CO_2 atmosphere. At 2, 6, and 12 h after infection, the medium was removed, and the cultures were washed once with 1 ml of methionine-free MEM. To each well, 0.2 ml of methionine-free MEM supplemented with 50 μCi (1 Ci = 37 GBq) of [^{35}S]methionine was added and incubated for 30 min at 37°C. Cytoplasmic extracts of infected cells were prepared by incubating each well in 0.2 ml of 0.5% Nonidet P-40 lysis buffer for 10 min at 37°C. For pulse–chase experiments, cells

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Abbreviations: CEF, chicken embryo fibroblast cells; FCS, fetal calf serum; MVA, modified vaccinia Ankara; pfu, plaque-forming unit(s); WT, wild-type virus.

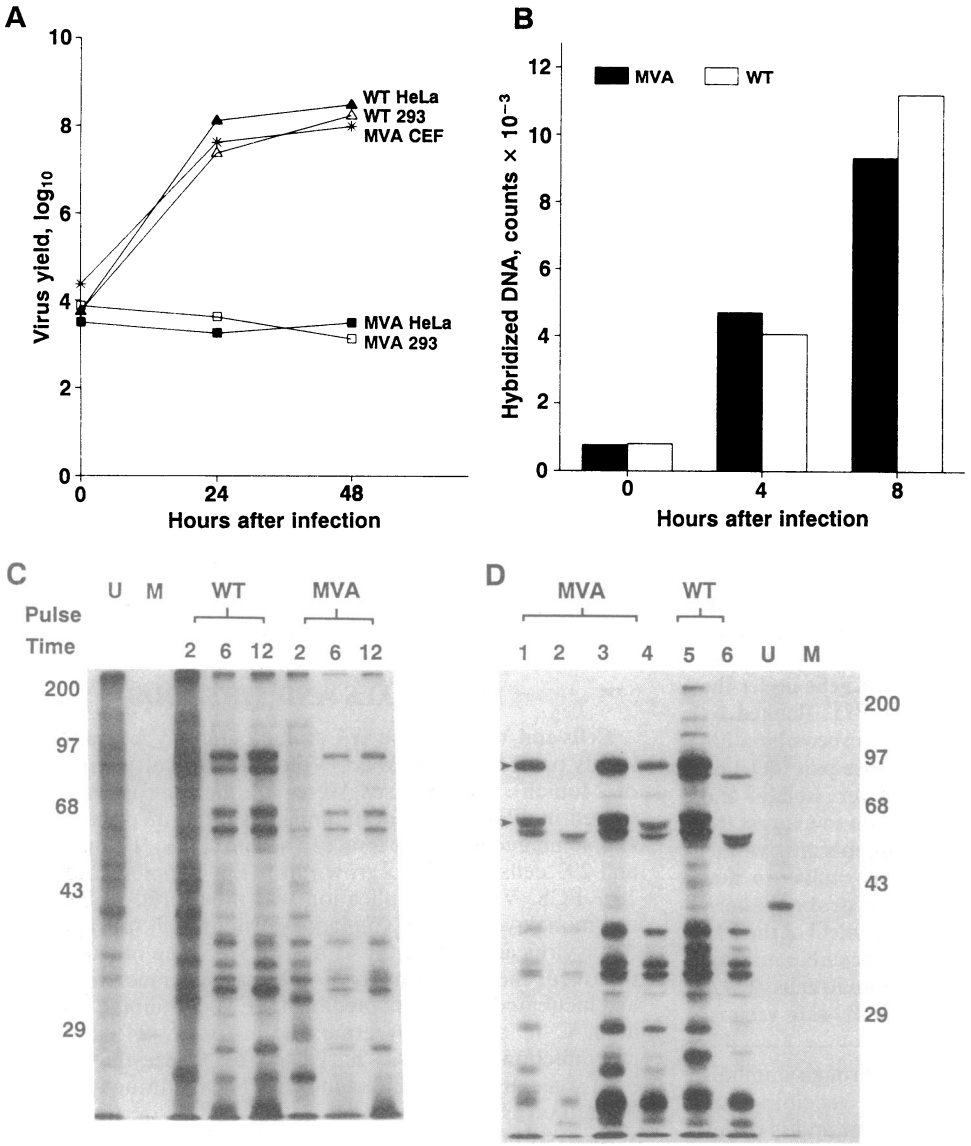

FIG. 1. Characterization of the infection of human cells with MVA. (*A*) Multiplication of MVA in HeLa (■), 293 (□), and CEF (*) cells and of WT in HeLa (▲) and 293 (△) cells. (*B*) Viral DNA synthesis was determined by hybridization of a ^{32}P-labeled viral DNA probe to DNA isolated from 293 cells at 0, 4, or 8 h after infection with MVA or WT. Radioactivity was quantitated using a Betascope 603 blot analyzer. (*C*) Synthesis of early and late viral polypeptides in human cells. HeLa cells were infected with WT or MVA and labeled with [^{35}S]methionine at the indicated hours postinfection (Pulse Time). Cell lysates were treated with 2% SDS and 1% dithiothreitol and analyzed by electrophoresis on a 10% polyacrylamide gel. Lane U, uninfected cell extract; lane M, protein standards (indicated by their molecular masses in kDa on the left). (*D*) Proteolytic processing of viral proteins. CEF (lanes 1 and 2) or HeLa (lanes 3–6) cells were infected with MVA or with WT for 6 h and labeled with [^{35}S]methionine for 30 min. Cells were harvested after the pulse (lanes 1, 3, and 5) or after a 16-h chase (lanes 2, 4, and 6). Cell lysates were treated with 2% SDS and 1% dithiothreitol and analyzed by electrophoresis on a 10% polyacrylamide gel. Lanes U and M are defined as in *C*, and the positions of the marker proteins (in kDa) are indicated on the right. The uncleaved precursors of the major late core polypeptides, P4a and P4b, are marked by arrowheads.

were infected for 6 h and then incubated in 0.2 ml of methionine-free MEM containing 50 μCi of [^{35}S]methionine for 30 min. The cells were subsequently washed once with MEM and incubated in MEM supplemented with 2% FCS for another 16 h. Cells were lysed as above, and samples were analyzed by SDS/PAGE.

Electron Microscopic Analysis. Cell monolayers were infected with virus at a multiplicity of 10 pfu per cell. At 16 h after infection, the medium was removed, and the cells were fixed by the addition of 2.5% (vol/vol) glutaraldehyde in Millonig's buffer (13). After 1 h of incubation at 4°C, the cells were scraped and then pelleted by low-speed centrifugation. The cell pellet was incubated an additional 30 min in the same 2.5% glutaraldehyde buffer at 4°C. Then the buffer was replaced by Millonig's 0.13 M sodium phosphate buffer (pH 7.4). Transmission electron micrographs were prepared by Advanced Biotechnology, Silver Spring, MD).

Plasmids. Sequences of MVA DNA flanking the site of a 3500-bp deletion in the *Hin*dIII A fragment of the MVA genome were amplified by PCR and cloned into pGEM 4Z (Promega). The primers for the left 900-bp DNA flank were 5′-CAGCAGGAATTCGTTGGTGGTCGCCATGGATG-GTGT-3′ and 5′-GGGGGGGGTACCTACCAGCCAC-CGAAAGAG-3′ (sites for restriction enzymes *Eco*RI and *Kpn* I are underlined). The primers for the right 600-bp DNA flank were 5′-GGGGGGCTGCAGTTTGGAAAGTTTTAT-AGG-3′ and 5′-GGGGGGAAGCTTAACTAGTTTCTG-GTG-3′ (sites for the restriction enzymes *Pst* I and *Hin*dIII are underlined). Between these flanks of MVA DNA, the *Escherichia coli lacZ* gene under control of the vaccinia virus late promoter P11 and the *E. coli gpt* gene under control of the vaccinia virus early/late promoter P7.5 were cloned.

Generation of Recombinant Viruses. CEF infected with MVA at a multiplicity of 0.05 pfu per cell were transfected with calcium phosphate-precipitated plasmid as described (14). Recombinant MVA virus expressing β-galactosidase (MVA LZ) was selected by six consecutive rounds of plaque purification in CEF stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (300 μg/ml) (15). After one blind passage on CEF in the presence of mycophenolic acid, recombinant MVA virus expressing β-galactosidase and guanine phosphoribosyltransferase (MVA LZgpt) was selected by three consecutive rounds of plaque purification on CEF in the presence of mycophenolic acid and 5-bromo-4-chloro-3-indolyl β-D-galactoside screening for blue virus plaques (16). Subsequently, viruses were amplified by infection of CEF monolayers, and the DNA was analyzed by Southern blot hybridization.

β-Galactosidase Assay. Confluent HeLa cells and CEF were infected with 15 pfu per cell of MVA LZ, MVA LZgpt, or vaccinia virus Western Reserve recombinant vSC8, which also expresses the β-galactosidase gene under control of

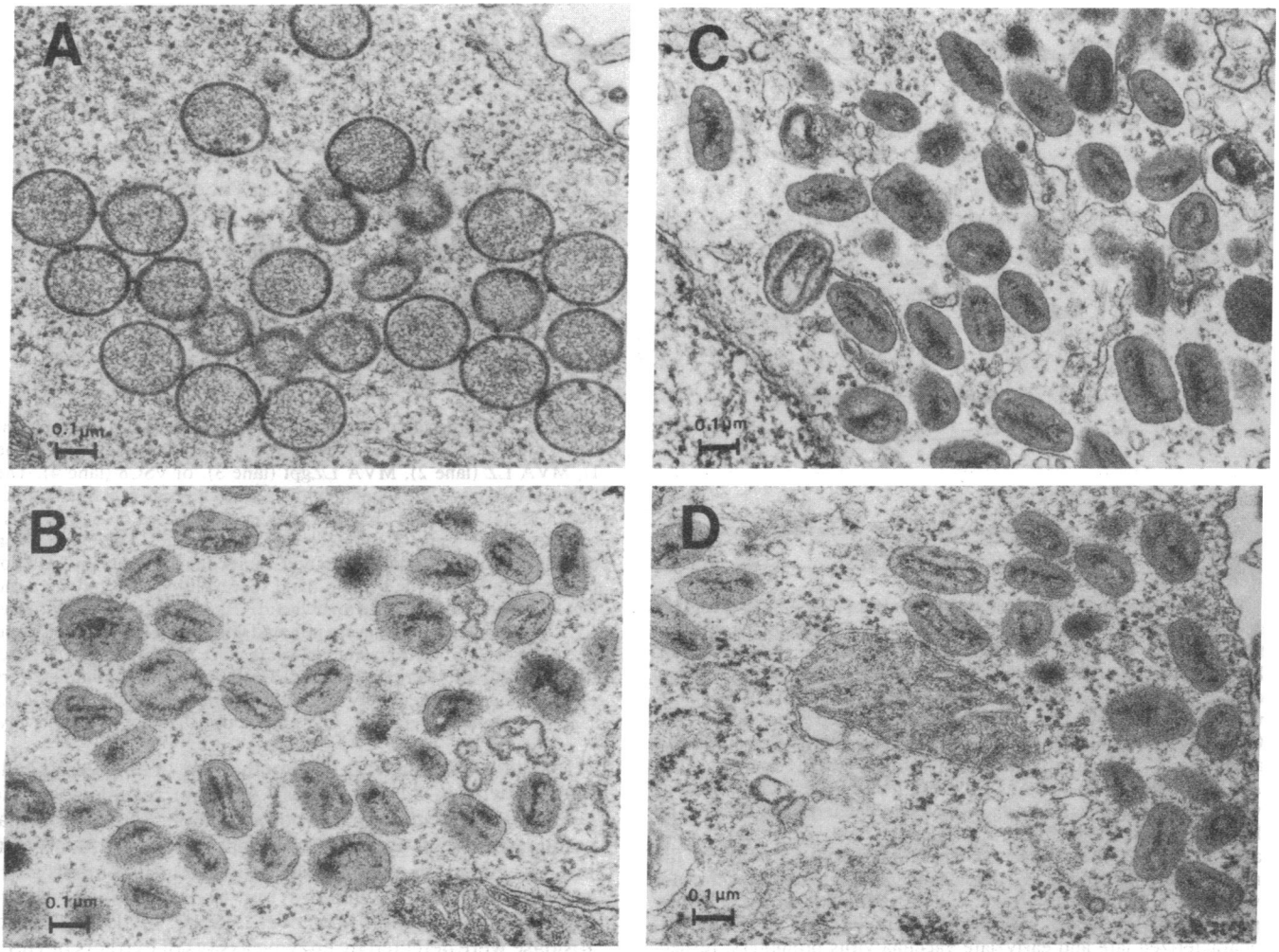

FIG. 2. Electron micrographs of infected cells. HeLa cells infected with MVA (*A*) or WT (*B*) and CEF cells infected with MVA (*C*) or WT (*D*) for 16 h were fixed, sectioned, and examined by electron microscopy. (Bars = 0.1 μm.)

vaccinia virus promoter P11 (15). After a 24-h incubation, cytoplasmic extracts were prepared, and the protein content (17) and the specific β-galactosidase activity (18) were determined.

RESULTS

Characterization of the Host-Range Defect of MVA in Human Cells. Previous work had shown that MVA is unable to multiply in a broad range of mammalian cell lines (7). In HeLa and 293 cells, WT increased 10,000-fold in titer, whereas no increase in MVA was detected (Fig. 1*A*). By contrast, MVA grew well in CEF (Fig. 1*A*), usually reaching a titer higher than that of WT (data not shown). Despite the differences in the abilities of WT and MVA to multiply in human cells, viral DNA replication proceeded similarly (Fig. 1*B*), and restriction enzyme analysis indicated that the concatemeric forms of replicative MVA DNA were processed normally to unit genomes (data not shown).

The replication of MVA DNA implied that the initial stages of infection comprising viral attachment, entry, early gene expression, and uncoating occurred in nonpermissive human cells. SDS/PAGE analysis of extracts of WT- and MVA-infected HeLa cells that had been pulse-labeled with [^{35}S]methionine confirmed the synthesis of early viral proteins and demonstrated that late viral proteins were made at 6 and 12 h after infection (Fig. 1*C*). The viral protein patterns in WT- and MVA-infected human cells were very similar. An exception, the absence of a 90,000-Da polypeptide from MVA-infected cells, was attributed to the deletion of the gene encoding the nonessential A-type inclusion protein homolog (7). This difference was also noted upon analysis of labeled proteins from WT- and MVA-infected permissive CEF.

Since pulse-labeling studies showed no significant difference in early or late viral protein synthesis, we checked to see if there was a defect in protein processing. When MVA-infected CEF were labeled with [^{35}S]methionine at 6 h after infection and then chased with unlabeled amino acids for 16 h, the expected cleavage of at least five polypeptides including the major core protein precursors, P4a and P4b, was observed (Fig. 1*D*). By contrast, processing of MVA proteins in HeLa cells was inhibited, whereas WT proteins were processed correctly (Fig. 1*D*).

Virus Morphogenesis in Infected Cells. From studies using the drug rifampicin, it is known that an interruption of virus assembly leads to the inhibition of proteolytic cleavage of the major core precursors P4a and P4b in infected cells (19). Similarly, when the expression of an 11,000-Da DNA binding protein of vaccinia virus is prevented, characteristic immature virus particles are found in infected cells, and proteolytic cleavage of P4a and P4b is blocked (20). Since proteolytic processing of late viral polypeptides was also inhibited in MVA-infected HeLa cells, we suspected that MVA virion formation failed to occur in nonpermissive human cells. Indeed, only immature virus particles appearing as circular spicule-coated membranes encircling granular material and

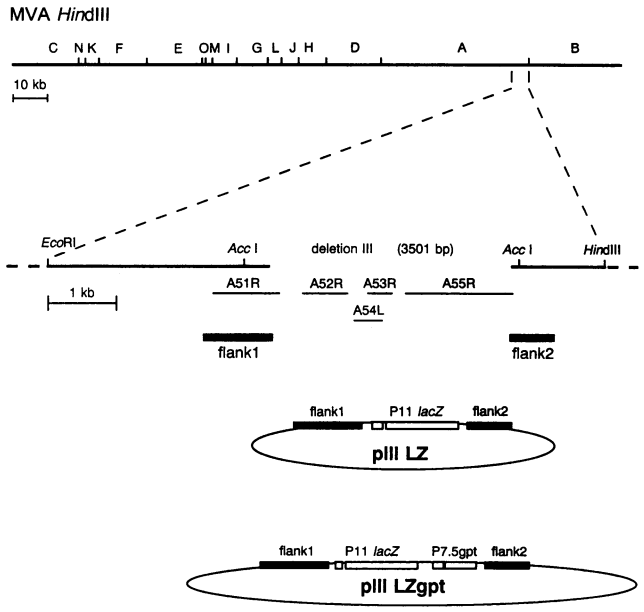

FIG. 3. Schematic map of the genome of MVA and plasmids designed for insertion of foreign DNA by homologous recombination. *Hin*dIII restriction endonuclease sites within the genome of MVA are indicated at the top. The 480-bp *Acc* I–*Acc* I fragment that overlapped the junction of deletion III within the *Hin*dIII A fragment was sequenced and compared to the published sequence of the Copenhagen strain of vaccinia virus (39). This comparison suggested a deletion of 3501 bp affecting the five open reading frames A51R, A52R, A53R, A54L, and A55R present in the Copenhagen strain of vaccinia virus but partially or completely deleted from MVA. The DNA sequences adjacent to the deletion (flank1 and flank2) were amplified by PCR and inserted into the left and right ends of the multiple cloning site of a pGEM plasmid in order to make the transfer vector pIII, which was used for the construction of the insertion plasmids pIII LZ and pIII LZgpt. P11 and P7.5 refer to well-characterized late and early/late vaccinia virus promoters, respectively. kb, kilobase(s).

lacking dense nucleoprotein bodies could be seen by electron microscopic examination of human cells infected with MVA (Fig. 2*A*). By contrast, mature brick-shaped particles with complex internal structures were numerous in human cells infected with WT (Fig. 2*B*) or CEF infected with MVA (Fig. 2*C*) or WT (Fig. 2*D*).

Construction and Isolation of MVA Recombinants Expressing the *E. coli lacZ* Gene. The robust synthesis of viral late proteins, despite the absence of infectious virus formation in mammalian cells, suggested that MVA might be a useful vector for expression of foreign genes. To test this strategy and monitor gene expression, the *E. coli lacZ* gene regulated by the well-characterized vaccinia virus late promoter P11 (21) was flanked by MVA DNA sequences to form the plasmid insertion vector pIII LZ (Fig. 3). The foreign gene was targeted precisely to the site of a deletion within the MVA genome to avoid any further changes in its phenotype. Recombinant vaccinia virus MVA LZ was formed in CEF that were infected with MVA and transfected with pIII LZ. Recombinant virus plaques were identified by screening for β-galactosidase synthesis with a chromogenic substrate (15). A second plasmid, pIII LZgpt, contained the *lacZ* gene as well as the *E. coli gpt* gene under the control of vaccinia virus early/late promoter P7.5 to provide antibiotic selection of recombinant virus MVA LZgpt (16). In each case, multiple plaque isolations were performed, and the correct insertion of the foreign DNA and absence of parental virus was ascertained by restriction enzyme analysis and Southern blot hybridization.

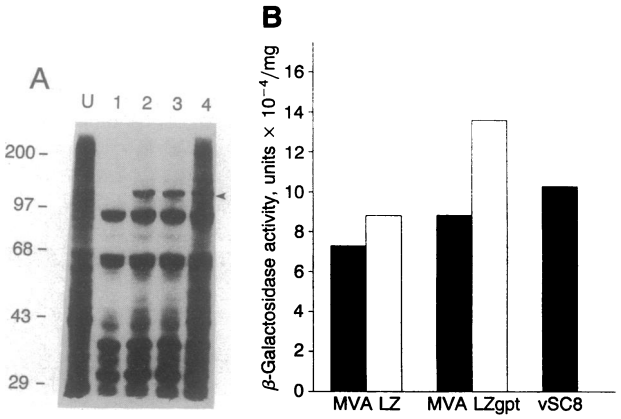

FIG. 4. Expression of the *E. coli lacZ* gene by MVA recombinant viruses. (*A*) SDS/PAGE of human 293 cells infected with MVA (lane 1), MVA LZ (lane 2), MVA LZgpt (lane 3), or vSC8 (lane 4). The infected cultures were labeled with [^{35}S]methionine at 6 h postinfection. Cell lysates were analyzed by electrophoresis on a 10% polyacrylamide gel. Lane U, uninfected 293 cell extract. The numbers on the left indicate the positions and molecular masses (in kDa) of protein standards. The protein band representing the enzyme β-galactosidase is marked by an arrowhead. (*B*) β-Galactosidase activity. CEF (□) or HeLa (■) cells were infected with MVA LZ, MVA LZgpt, or vSC8 for 24 h. Cytoplasmic extracts were prepared, and β-galactosidase activities were determined. The 0 time values, obtained from extracts made immediately after infection with MVA LZ, MVA LZgpt, and vSC8 were 178, 132, and 61 units per mg, respectively.

Metabolic labeling with [^{35}S]methionine of human 293 cells infected with MVA LZ and MVA LZgpt revealed the late synthesis of an additional protein of about 116,000 Da that comigrated with β-galactosidase made by vSC8 (15), a Western Reserve strain recombinant virus that also contains a P11 promoter–*lacZ* gene cassette but that multiplies well in human cells (Fig. 4*A*). Moreover, similar levels of β-galactosidase were detected by enzyme assays of extracts of HeLa cells infected with the MVA and Western Reserve strain recombinant viruses (Fig. 4*B*).

DISCUSSION

The structural and other abundant proteins made by vaccinia virus are products of the late class genes, which are expressed only after viral DNA replication (23). The high level of protein synthesis achieved with recombinant MVA in nonpermissive human cells is consistent with our finding that the block in virus assembly occurs after DNA replication. This desirable result was not predicted because a large number of open reading frames, including the K1L host-range gene, are impaired in MVA (ref. 7; G.S., unpublished data). Previous studies had indicated that a deletion including the K1L gene from the Copenhagen strain of vaccinia virus resulted in abortive expression of early viral genes followed by a rapid inhibition of further viral and cellular protein synthesis (11, 24). In addition, viral DNA replication was inhibited, and there was no evidence of viral membranes or immature particles in the nonpermissive human cells infected with the K1L mutant (11). Viral DNA replication was also severely inhibited in human cells infected with NYVAC, a genetically engineered virus derived for recombinant vaccine purposes from the Copenhagen strain by deletion of multiple open reading frames including K1L and a second host-range gene, C7L (25). Furthermore, naturally host range-restricted avipoxviruses cannot carry out DNA replication in mammalian cells (25). Nevertheless, the results obtained with MVA are not entirely unprecedented since some host-range white pock deletion mutants of rabbit poxvirus support DNA replication and expression of late proteins in nonpermissive

pig kidney cells (26). The rabbit poxvirus mutants, however, exhibited quantitatively decreased late protein synthesis in nonpermissive cells and were blocked at a stage after the proteolytic processing of core polypeptides (26). Thus, the host-range phenotype of MVA appears to be unique. In view of the extensive deletions in MVA, further investigations are needed to determine the genetic defects primarily responsible for its phenotype. Since MVA still cannot multiply in human cells after introduction of an intact K1L gene, the host-range phenotype must be multifactorial (ref. 7; G.S., unpublished data).

LC16mO, an attenuated strain of vaccinia virus that still replicates well in mammalian cells, including animal skin, has been used to express foreign genes (27). In addition, a variety of gene deletions that attenuate vaccinia virus to varying degrees have been described, and some of these, individually and in combination, have been used for construction of expression vectors (25, 28–35). Avipox or other naturally host-restricted poxviruses also have been advocated and tested as nonreplicating vectors (22, 36–38). However, for use as a gene expression vector, no other characterized naturally derived or genetically engineered strain of poxvirus is known to have a similar combination of desirable properties and to have been so extensively tested in humans as MVA. The properties of the MVA strain of vaccinia virus that make it so attractive as a vector include (*i*) high-level gene expression in human and other mammalian cells, (*ii*) ability to prepare high titer virus stocks in primary and established ATTC CRL 1590 (G.S., unpublished data) CEF, (*iii*) inability to produce infectious virus in most mammalian cells, (*iv*) avirulence in a variety of animals even under immunosuppressive conditions, and (*v*) little or no local or systemic reaction upon inoculation of humans including high-risk individuals. Adoption of MVA-based vectors should reduce the risk of infecting laboratory workers. Whether MVA vectors will also be useful for live vaccine or therapeutic applications remains to be determined.

We thank S. Isaacs and M. Buller for discussions and comments on the manuscript. G.S. is supported by an AIDS Scholarship from the Bundesministerium fur Forschung und Technologie, Federal Republic of Germany.

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